ARS CFDA NO. 10.001

Budget Object Class Code

UNITED STATES DEPARTMENT OF AGRICULTURE
EXTRAMURAL AGREEMENT

TYPE OF RESEARCH AGREEMENT
Individual Memorandum of Understanding

TITLE OF PROJECT

The Effects of Mineral Applications on Phytonutrient Content of Plants

AGREEMENT NO.	TYPE OF ACTION
58-1235-1M-005	New

PERIOD OF AGREEMENT
1-May-01 thru 30-Apr-02

FEDERAL OBLIGATION	CHANGE IN FEDERAL OBLIGATION
N/A	☐ ☐

LOG NO.	AUTHORITY
N/A	7 U.S.C. 3318

OBLIGATION DISTRIBUTION

Accounting Code	Amount
N/A	$0.00

AGENCY (Name and Address)

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APPLICABLE PROVISIONS AND REGULATIONS

This Agreement includes the following:

- ■ **Statement of Work; or**
- ☐ **Project Summary; or**
- ☐ **Proposal; and**
- ☐ **Budget (Form ARS-454/455)**
- ☐ **USDA Civil Rights Poster (Form AD-475A); and**

Provisions:

- ☐ **General (Form ARS-452)**
- ☐ **General (Form REE-22)**
- ☐ **Special (Form ARS-453)**

Regulations:

- ☐ **7 CFR 3015.205 (by reference)**
- ☐ **7 CFR 3016 (by reference)**
- ☐ **7 CFR 3019 (by reference)**
- ■ **7 CFR 3015.175 (b), Copyrights (by reference)**
- ■ **37 CFR Part 401.14, Patents and Inventions (by reference)**

Appendices and Schedules:

- ☐ **Appendix A - Application for Funding (ARS-403)**
- ☐ **Appendix B - Current and Pending Federal Financial Assistance Support (ARS-408)**
- ☐ **Appendix C - Research Assurance Statement (ARS-411)**
- ☐ **Appendix D - Civil Rights Assurance Certification (ARS-405)**
- ☐ **Appendix E - Civil Rights Questionnaire (ARS-406)**
- ☐ **Appendix F - Certification Regarding Debarment, Suspension and other Responsibility Matters - Primary Covered Transactions (AD-1047)**
- ☐ **Appendix G - Certification Regarding Debarment, Suspension and other Responsibility Matters - Lower Tier Covered Transactions (AD-1048)**
- ☐ **Appendix H - Certification Regarding Drug-Free Workplace Requirements - Non-Individuals (AD-1049)**
- ☐ **Appendix I - Certification Regarding Drug-Free Workplace Requirements - Individuals (AD-1050)**
- ☐ **Appendix J - Certification/Disclosure Requirements Related to Lobbying (SF-LLL)**
- ☐ **Appendix K - Intellectual Property Rights (Applicable to International Agreements only)**
- ■ **Appendix L - Report of Inventions and Subcontracts**

Other (Specify):

- ■ **Form ARS-451 - page 2** (Organization Certification)
- ■ **ADODR Instructions**

Payment: N/A

- ☐ **HHS/Payment Management System**
- ☐ **Treasury Check/EFT**
- ☐ **Advance Payment Authorized**
- ☐ **Pre-Award Costs Authorized (See Below)**

Reporting Requirements:

Submit:

☐ Performance Reports	☐ Financial Reports
☐ Quarterly	☐ Quarterly
☐ Semi-annual	☐ Semi-annual
☐ Annual	☐ Annual
☐ Final	☐ Final

- ☐ **Annual Report of Inventions and Subcontracts**

To:

- ☐ **ADODR**
- ☐ **Sponsoring Organization**

FOR THE UNITED STATES DEPARTMENT OF AGRICULTURE

AUTHORIZED DEPARTMENTAL OFFICER	TYPED NAME	DATE
Michael A. Wyckoff	MICHAEL A. WYCKOFF	6-5-01

FOR THE SPONSORING ORGANIZATION
(Signature of persons authorized to incur contractual obligations)

SIGNATURE	TYPED NAME AND TITLE	DATE
B W Ferguson	BRUCE W. FERGUSON, PRESIDENT & CEO	MAY 23, 2001
SIGNATURE	TYPED NAME AND TITLE	DATE

EFFECT OF DIVALENT CATIONS ON THE PHENOLIC ACIDS AND FLAVONOL GLYCOSIDES OF LETTUCE (*LACTUCA SATIVA* L.) LEAF TISSUES

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ABSTRACT

The effect of calcium (Ca(II)), copper (Cu(II)), iron (Fe(II)) or manganese (Mn(II)) on the phenolic compounds of green leaf lettuce (*Lactuca sativa* L. cv Simpson Elite) was investigated. Leaf disks (7 mm) were exposed to divalent cation concentrations ranging from 50 μM to 2.5 mM at pH 5.0 for 24 hr in both the light and dark. Acidified, aqueous methanol (flavonoid-rich fraction) extracts were analyzed by reverse-phase, high performance liquid chromatography. Cu(II) and Fe(II) altered the levels of the phenolic compounds, increasing the levels of some compounds at low concentrations and decreasing the levels of all the phenolic compounds at higher levels. At low concentrations, Cu(II) and Fe(II) stimulated the production of dicaffeoylquinic acid which formed in response to tissue wounding under both light conditions. These cation-induced changes in phenolic compounds may modify plant responses to environmental or biotic stresses and alter the nutritional values of certain vegetable crops.

INTRODUCTION

Many of the reported human health benefits of leafy vegetables have been attributed to the presence of phenolic compounds in the leaf tissues (1,2). In previous studies (3-6), the effect of elevated manganese (Mn(II)), iron (Fe(II)) or copper (Cu(II)) on the phenolic composition of cucumber and spinach leaf tissues was determined using a simple system of small leaf disks and 24 to 48 hr exposures to different divalent cation concentrations. Mn(II) produced few changes in the flavonoid composition of an aqueous methanol extract of the leaf tissues. Conversely, both Fe(II) and Cu(II) markedly altered the flavonoid composition of both cucumber and spinach leaf tissues. However, spinach and cucumber leaves contain primarily flavonol glycosides with few

phenolic acids (3,6). Therefore, the effects of elevated mineral content on plant leaf phenolic acids remain unclear. Since lettuce leaves contain large amounts of phenolic acids (7), a study was undertaken to determine whether increased leaf Ca(II), Cu(II), Fe(II) or Mn(II) could modify the level of both phenolic acids and flavonoids in leaf tissues of greenhouse-grown leaf lettuce.

MATERIALS AND METHODS

Plant Material, Sample Preparation and Treatment Procedures

Leaf disks (7 mm) were cut randomly with a number 2 cork borer from the leaves of 24-day-old greenhouse-grown lettuce (*Lactuca sativa* L. cv Simpson Elite) (Johnny's Selected Seeds, Albion, Maine). Sets of 15 leaf disks were floated, adaxial side down, on 5 ml of distilled water, containing 0, 0.05, 0.1, 0.25, 0.5, 1.0 or 2.5 mM Ca(II), Cu(II), Fe(II) or Mn(II), as their sulfate salts, at pH 5 in multiple 6-well tissue culture plates. To insure a random sampling of the lettuce plants, leaves were selected from different plants and 56 discs prepared. Single disks were then transferred to each of the 56 treatment solutions. This process was repeated until each well contained 15 disks. As this process required over 1 hr, the divalent cations were added to the treatment solutions after the disks were prepared. After wrapping one set for each treatment in foil (dark treatment), the culture plates were placed in a growth chamber at 27°C and exposed to 8 hr dark and then a 16 hr light.

Pigment Extraction Procedures

At the end of the 16 h photoperiod, the samples were taken to a laboratory equipped with a green safelight. To prepare triplicate samples, 5 leaf disks for each treatment were washed with distilled water, blotted dry and ground with a glass homogenizer in 500 µL of cold (-20°C) 40% (v/v) methanol, containing 1% (v/v) formic acid. After storage in the dark overnight at 4°C, the homogenized tissues were centrifuged at 14000*g* for 5 min and the supernatants stored at -80°C.

HPLC Analytical Procedures

All HPLC procedures were performed with a Waters 600 series pump and controller, and a Waters 990 photodiode array detector. For analysis of the aqueous methanol extracts, 200 µL of the extract was injected into a 25 cm x 4.6 mm Spherisorb ODS-2 (octadecylsilyl, 5µm particle

size) column (Sigma Chemical Company, St Louis. MO) and eluted with a 30 min linear gradient from 25% to 60% (v/v) methanol in 0.1% (v/v) aqueous formic acid. Spectra from 250 to 500 nm were stored, and the separation monitored at 340 nm.

After conversion of chromatograms or spectra into ASCII text files using the Waters 990 software, a series of WordPerfect macros were used to modify the ASCII files into Sigma Plot compatible format. All chromatograms represent data obtained at 1 s intervals and all spectra have 0.2 nm resolution. Statistical analyses were performed using the resident SigmaPlot column statistics. Unless otherwise noted, the values are expressed on an absolute basis, that is, total integrated absorbance units for equal areas extracted.

RESULTS AND DISCUSSION

Visual symptoms of mineral toxicity in plants often include the formation of chlorotic and necrotic leaf lesions (8,9). In the present study, except for discoloration at the edges of the leaf disks at Cu(II) and Fe(II) levels above 0.5 mM, no classical symptoms of mineral toxicity were apparent in the leaf disks after 24 h of treatment. However, at divalent cation concentrations of 1.0 and 2.5 mM, all treatment conditions produced a faint yellow coloration of the treatment solutions, suggesting a loss of leaf components to the solution. This observation is consistent with cation-induced changes in leaf membrane permeability (10).

Identification of the Aqueous Methanol-Extractable Lettuce Leaf Phenolic Compounds

Homogenization of fresh lettuce leaf tissues in acidified 40% (v/v) methanol resulted in the extraction of a variety of UV-absorbing compounds (Fig. 1) with spectral characteristics consistent with either flavonol glycosides (Figure 2D, K) or phenolic acids (Figure 2A, F, B, H).. Using similar extraction procedures, the primary UV light-absorbing compounds from lettuce separated by HPLC have been identified (11,12). In lettuce purchased at a local grocery, the primary leaf flavonol glycoside may be quercetin 7-glucoside 3-malonylglucoside (Figure 1A, peak d). However, consistent with other reports, the primary flavonol glycoside in the greenhouse-grown, leaf lettuce was quercetin 3-malonylglucoside (Figure 1, peak K). Unlike many other leafy vegetables which contain high levels of flavonoids (1,13), the primary UV-absorbing components in the leaf lettuce extracts are phenolic acids. Considering the published analyses (11,12), phenolic acid standards and known lettuce responses to wounding (14), the major phenolic acids in these lettuce leaf extracts were identified as caffeoyltartaric acid (CTA,

peak A), caffeoylquinic acid (CQA, peak B), dicaffeoyltartaric acid (DCTA, peak F) and dicaffeoylquinic acid (DCQA, peak H). CQA and DCQA are also known as chlorogenic and isochlorgenic acids, respectively. CTA and DCTA are also referred to as caftaric and chicoric acids, respectively. Quercetin standards eluted at about 28 min under these HPLC conditions. Therefore, none of the treatment employed in this study resulted in flavonol glycoside hydrolysis.

Effect of Divalent Cations on the Aqueous Methanol-Extractable Lettuce Leaf Phenolic Compounds

Comparing the results presented in Figure 1 with those found in Figures 3 through 6, it is apparent that DCQA levels were significantly increased by tissue wounding. This result is consistent with earlier reports (12,14) and confirms the identification of this compound in the tissue extracts. The chromatograms presented in Figures 3 through 6 also show clear differences in the ability of the different cations to modify the concentrations of the lettuce leaf phenolic compounds. Employed at higher concentrations to retard the post-harvest phenolic browning of lettuce stems (7,15), calcium primarily increased the levels of the phenolic compounds. At low concentrations, calcium increased the amounts of the flavonol glycosides in light-treated tissues over 200% relative to the controls (Fig. 7D,E). Although the dose-response relations for the effects of calcium on the phenolic acids were more complex, calcium clearly increased the wound-induced levels of DCQA in the light-treated tissues (Fig. 7C).

Consistent with similar studies using cucumber and spinach leaves (4-6), the higher concentrations of both Cu(II) and Fe(II) reduced the levels of all the lettuce leaf phenolics (Figures 4,5,7,8). In general, the changes induced by Cu(II) and Fe(II) were light independent. At the lower mineral concentrations, both Cu(II) and Fe(II) increased the levels of certain phenolics. In particular, DCQA levels were increased by about 200% and 250% by 100 μM Cu(II) or Fe(II), respectively (Figs. 7H, 8C). At similar concentrations, the amount of quercetin 3-malonylglucoside was also increased by Cu(II) and Fe(II) treatments (Figs. 7J, 8E). A similar effect of Cu(II) and Fe(II) on flavonol glycosides was obtained with cucumber leaf tissues. Since the cation-induced elevation in flavonol glycoside levels occurred at lower concentrations in the light relative to tissue treated in the dark, the total oxidative status of the tissues may be a factor in the production of flavonol glycosides by oxidative minerals.

Although primarily concerned with the effects of Mn(II) on carotenoid levels of cucumber leaf, a previous study suggested Mn(II) modifies the levels of a wound-induced phenolic acid in plant leaf tissues (3). Therefore, the ability of Mn(II) to modify the levels of wound-induced

DCQA was examined. As shown in Figs. 6 and 8H, Mn(II) had little effect on DCQA levels. However, Mn(II) increased the levels of flavonol glycosides in the light-treated tissues (Fig. 8I, J). Since this response was similar to that produced by Ca(II) (Fig. 7J, K) and both divalent cations can stabilize membrane structure, it is possible that Mn(II) and Ca(II) modify phenolic levels partially through their interactions with membranes.

CONCLUSIONS

The results of this study combined with previous investigations clearly indicate the ability of selected divalent cations to modify rapidly the phenolic content of plant leaf tissues (3-6). Since the leaf levels of phenolic acids and flavonol glycosides are altered by environmental conditions (16-18), mineral-induced changes in these phytochemicals could modify plant responses to both biotic and abiotic stresses. Furthermore, these results suggest that the levels of selected phenolic acids and flavonol glycosides might be increased by mineral treatments. Vegetables grown in greenhouses or controlled environment chambers often have reduced levels of leaf phenolics. Since many of the nutritional benefits of vegetables have been attributed to the antioxidative or antimicrobial activities of phenolic acids and flavonoids (19-22), foliar mineral applications or modified nutrient solutions could enhance the phytonutritional quality of vegetable crops.

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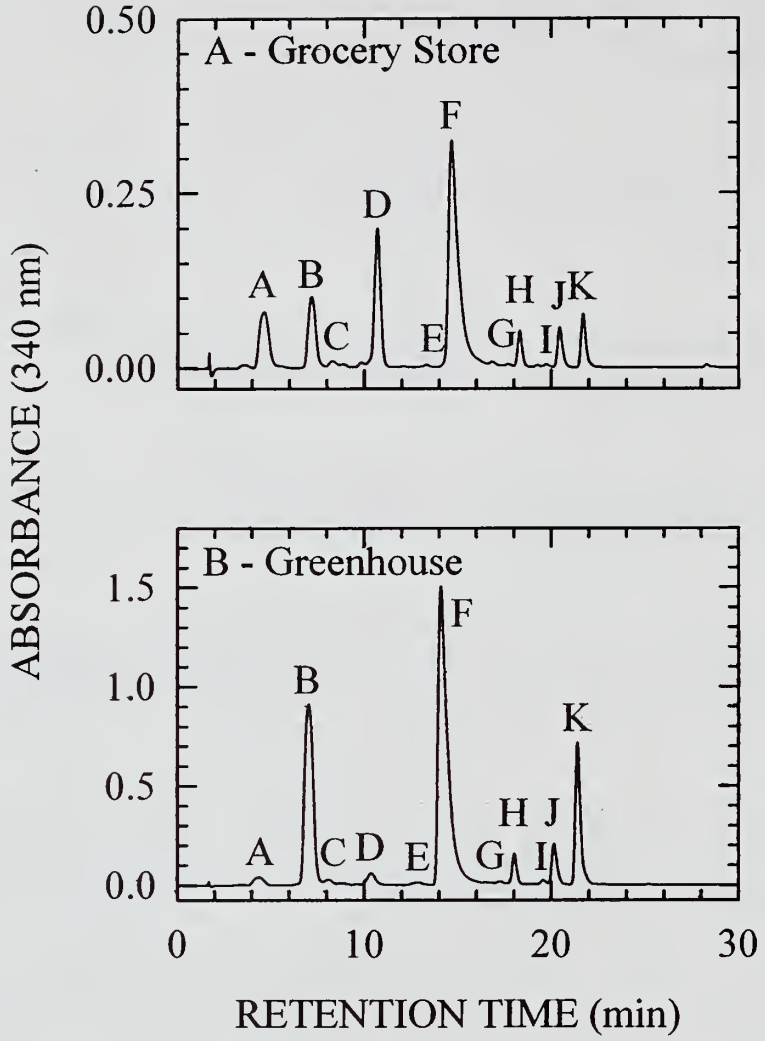

FIGURE 1. HPLC chromatograms of the phenolic compounds in acidified, methanol extracts of commercially- and greenhouse-grown green leaf lettuce. The absorbance was recorded at 340 nm Selected peaks are labeled with the same lower-case letters in both sections. Some of the major components have been identified: A. caffeoyltartaric acid (caftaric acid); B. caffeoylquinic acid (chlorogenic acid); F. dicaffeoyltartaric acid (chicoric acid); H. dicaffeoylquinic acid (isochlorogenic acid); K. quercetin 3-malonylglucoside.

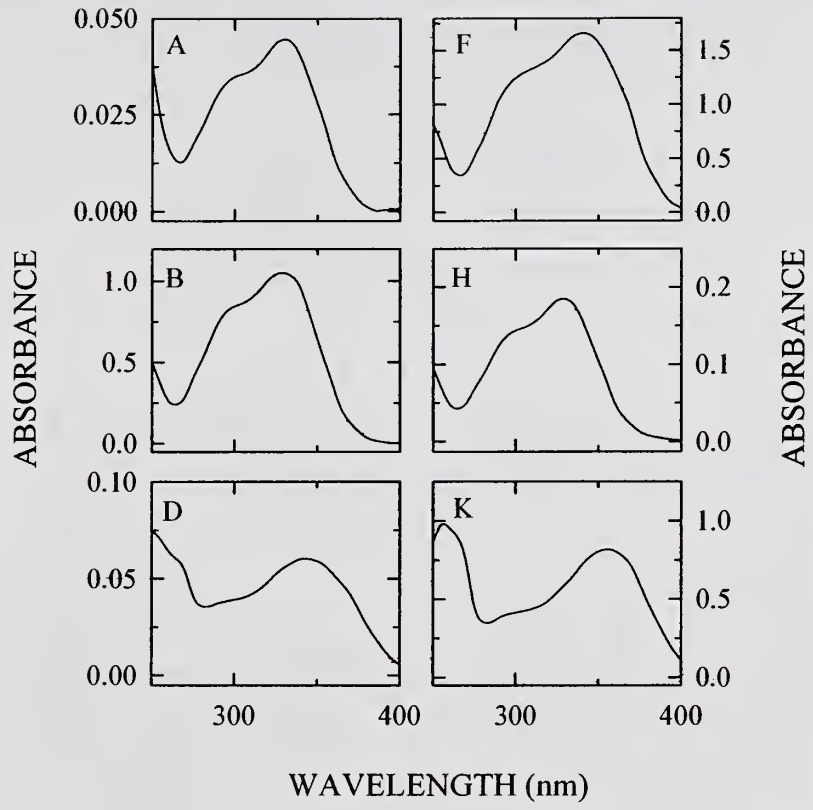

FIGURE 2. Spectra of selected lettuce-leaf phenolic compounds separated by HPLC. The sections are labelled with the letter peak designations given in Figure 1.

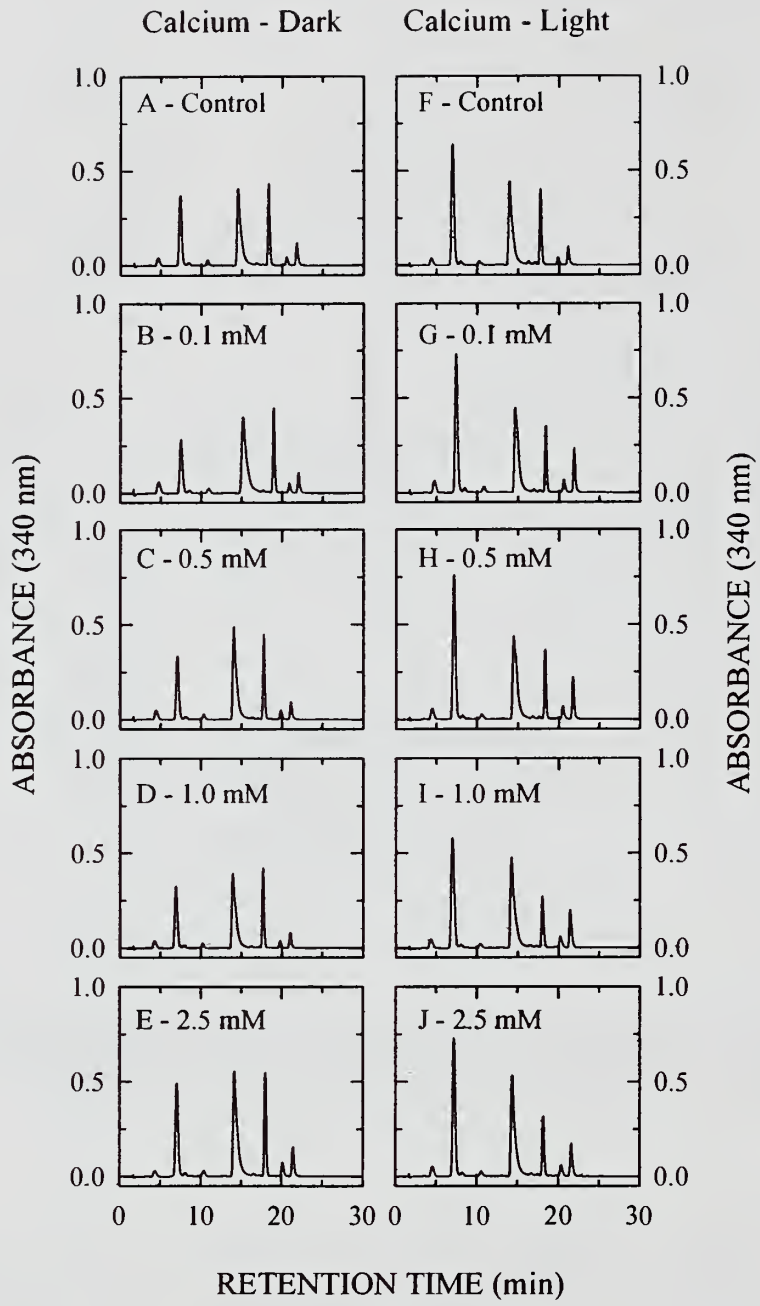

FIGURE 3. Representative HPLC chromatograms (340 nm absorbance) of acidified, methanol extracts from lettuce leaves treated with the given concentration of Ca(II) in distilled water at 5.0 in the dark or light (16 h photoperiod) for 24 h. The depicted chromatograms were random selections from the triplicate analyses of each treatment.

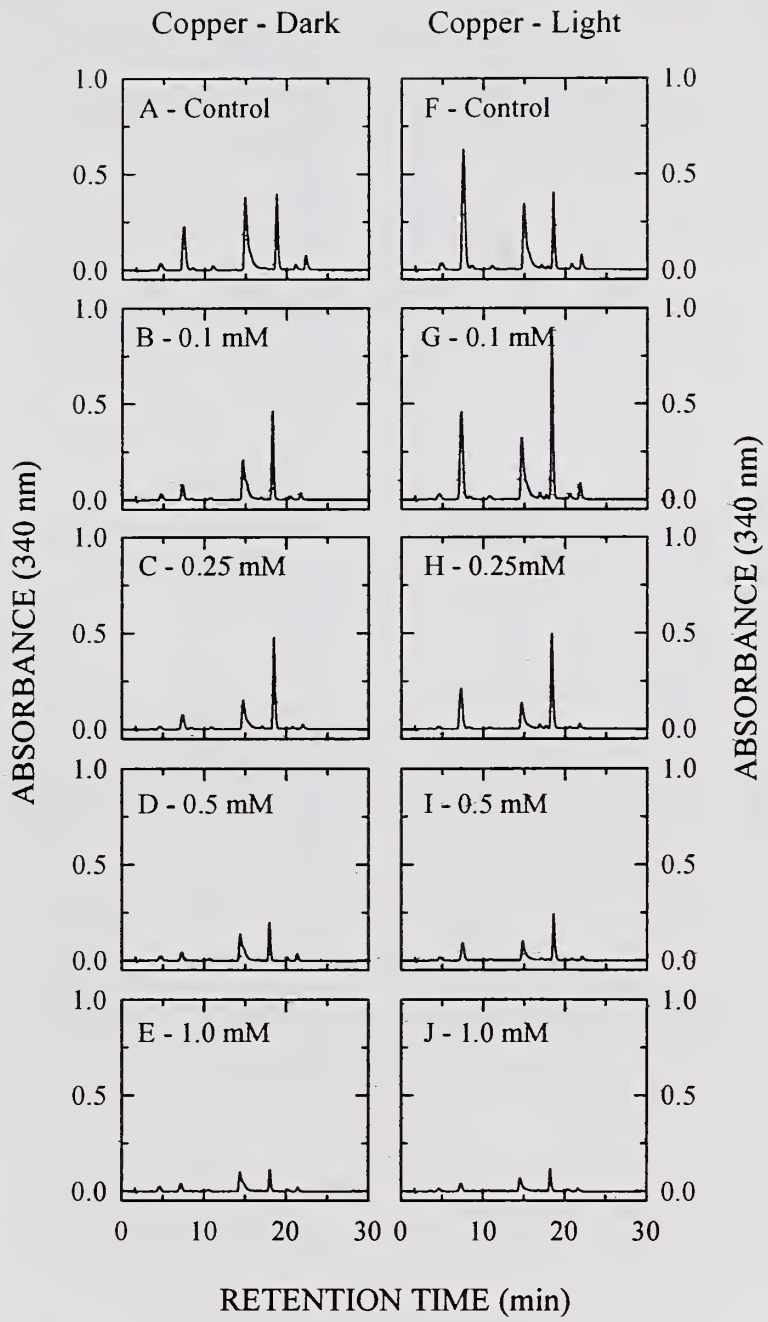

FIGURE 4. Representative HPLC chromatograms (340 nm absorbance) of acidified, methanol extracts from lettuce leaves treated with the given concentration of Cu(II) in distilled water at 5.0 in the dark or light (16 h photoperiod) for 24 h. The depicted chromatograms were random selections from the triplicate analyses of each treatment.

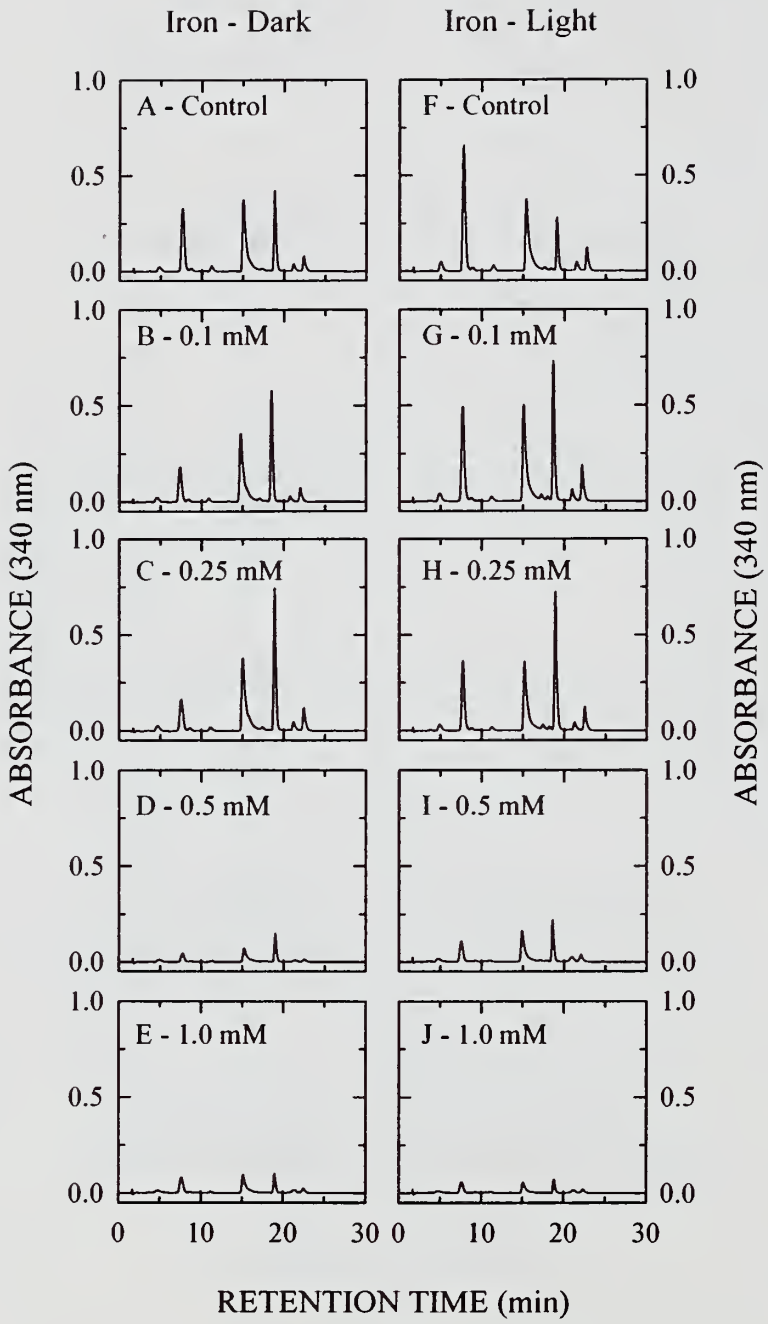

FIGURE 5. Representative HPLC chromatograms (340 nm absorbance) of acidified, methanol extracts from lettuce leaves treated with the given concentration of Fe(II) in distilled water at 5.0 in the dark or light (16 h photoperiod) for 24 h. The depicted chromatograms were random selections from the triplicate analyses of each treatment.

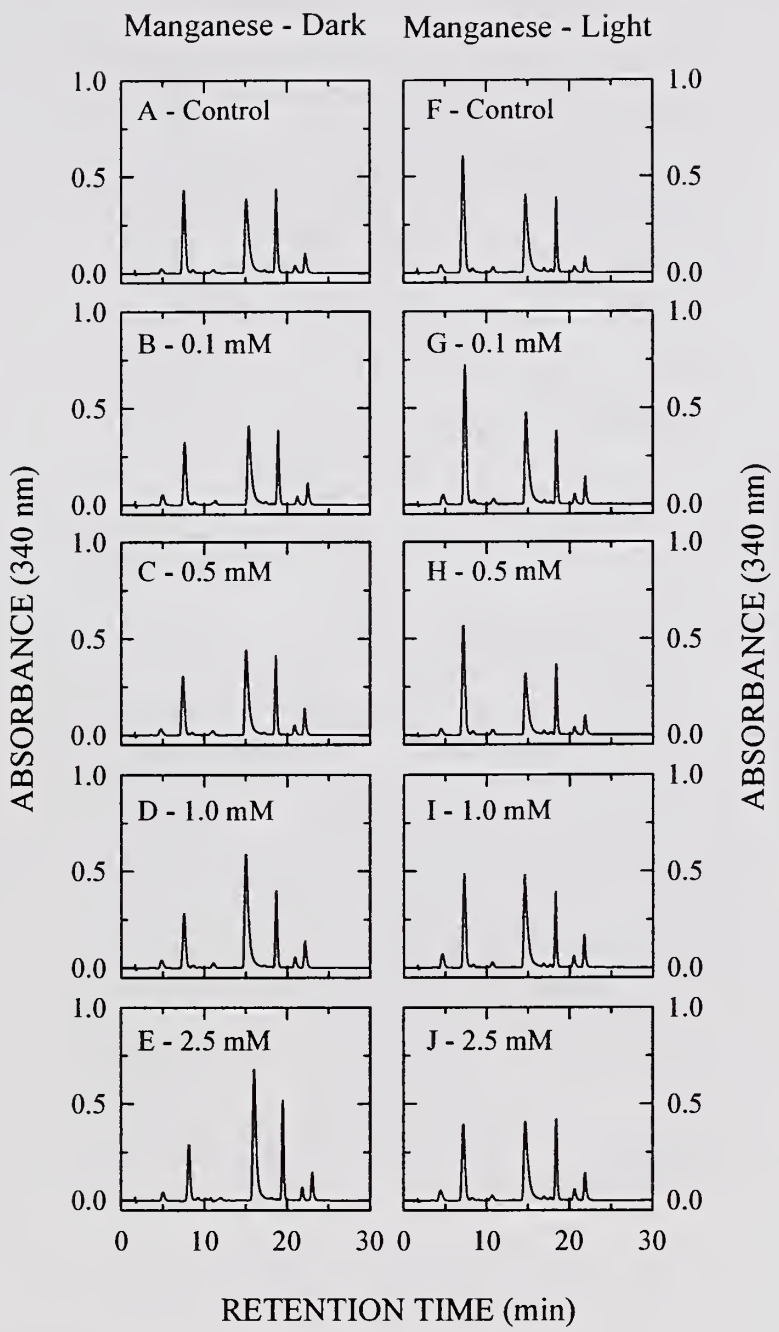

FIGURE 6. Representative HPLC chromatograms (340 nm absorbance) of acidified, methanol extracts from lettuce leaves treated with the given concentration of Mn(II) in distilled water at 5.0 in the dark or light (16 h photoperiod) for 24 h. The depicted chromatograms were random selections from the triplicate analyses of each treatment.

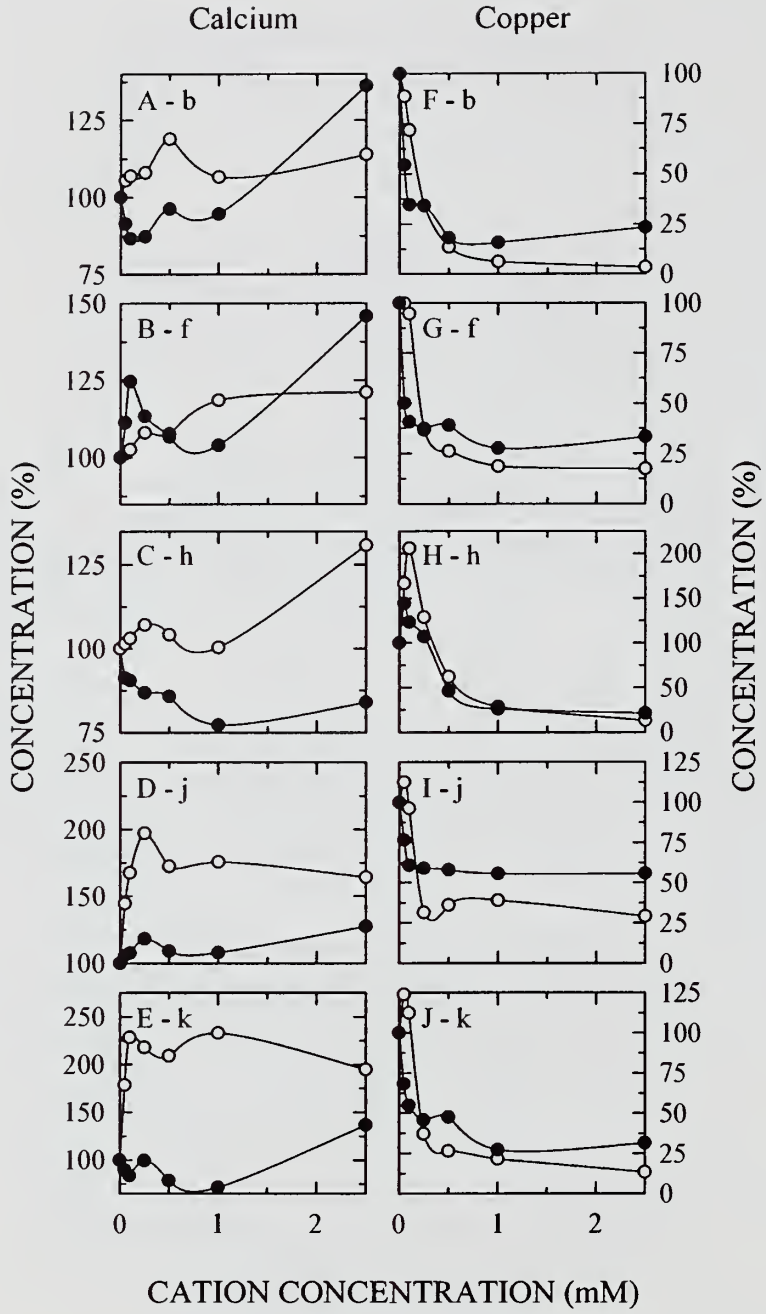

FIGURE 7.Relative Ca(II)- and Cu(II)-induced changes in selected lettuce leaf compounds separated by HPLC. The lower-case letter in each section label refers to the peak designations given in Figure 1.The results are presented as a percentage of the control values obtained in the absence of added cation and in the light (○) or in the dark (●). Each value is the mean of triplicate determinations and the average standard error of the mean was less than 3%.

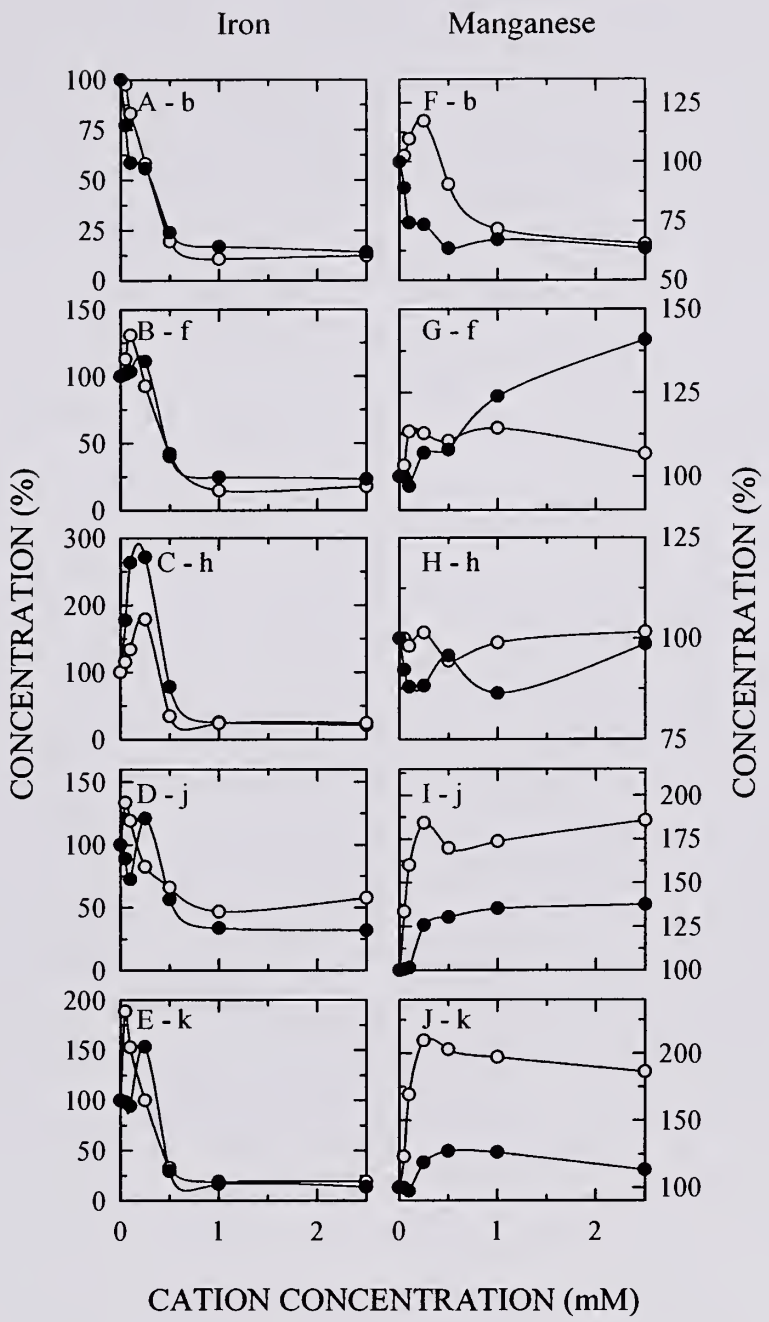

FIGURE 8.Relative Fe(II)- and Mn(II)-induced changes in selected lettuce leaf compounds separated by HPLC. The lower-case letter in each section label refers to the peak designations given in Figure 1.The results are presented as a percentage of the control values obtained in the absence of added cation and in the light (○) or in the dark (●). Each value is the mean of triplicate determinations and the average standard error of the mean was less than 3%.